



Material de trabajo del Curso de Posgrado

RT - qPCR

**Diseño, ejecución y
reporte de experimentos
de retrotranscripción
seguida de reacción en cadena
de la polimerasa cuantitativa
en tiempo real**

**Escuela de Graduados
Facultad de Ciencias Bioquímicas y Farmacéuticas
Universidad Nacional de Rosario**

MATERIAL DE TRABAJO DEL CURSO DE POSTGRADO

DISEÑO, EJECUCIÓN Y REPORTE DE EXPERIMENTOS DE RETROTRANSCRIPCIÓN SEGUIDA DE REACCIÓN EN CADENA DE LA POLIMERASA CUANTITATIVA EN TIEMPO REAL RT-qPCR

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**ESCUELA DE GRADUADOS DE LA FACULTAD DE CIENCIAS BIOQUÍMICAS Y FARMACÉUTICAS
UNIVERSIDAD NACIONAL DE ROSARIO**

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Diseño, ejecución y reporte de experimentos de retrotranscripción seguida de reacción en cadena de la polimerasa cuantitativa en tiempo real RT-qPCR. - 1a ed. - Rosario : Ediciones DelRevés, 2014.

58 p. ; 29x21 cm.

ISBN 978-987-29098-4-0

1. Biotecnología. I. Título
CDD 664.028

Fecha de catalogación: 06/02/2014

Ilustración de Tapa: *Juanito Laguna remontando su barrilete*, Antonio Berni,
192 x 109 cm.

La Guía de actividades de laboratorio fue confeccionada por María Noelia Lardizábal y Luis Veggi. Agradecemos los aportes y correcciones realizados por María Florencia Ércoli, y Carla Schommer.

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PREFACIO

En los últimos años, la técnica de Transcripción Reversa seguida de una Reacción en Cadena de la Polimerasa Cuantitativa (RT-qPCR) se convirtió en una metodología de referencia para determinar los niveles de expresión génica debido a su precisión, sensibilidad, exactitud, especificidad, reproducibilidad y robustez. Además de su óptima performance analítica presenta una amplia variedad de aplicaciones lo que en conjunto determinó su amplia y rápida difusión. Sin embargo para el usuario novato se presenta como una metodología difícil de utilizar fundamentalmente debido a la complejidad metodológica en la ejecución y en el procesamiento de los datos. En un intento por organizar el estudio que permita la mejor difusión y desarrollo de la técnica la comunidad científica internacional generó un consenso sobre la información necesaria para realizar la misma denominado The MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments). Nuestro objetivo es poder brindarles herramientas y asesoramiento para que a lo largo del curso basados en la propuesta de la Guía MIQE desarrollen un protocolo posible de RT-qPCR que permita la puesta a punto o la optimización de una determinación. Para esto los docentes del curso expondremos nuestras experiencias y conocimientos en diferentes aspectos y aplicaciones de la técnica que como resultado de nuestra tarea cotidiana hemos ido adquiriendo y nos sentimos felices de compartir abiertamente avalando el espíritu de las instituciones publicas a la que pertenecemos. Desde ya esperamos les sea de utilidad y les quede un buen recuerdo de los momentos juntos.

Luis Veggi
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GUÍA DE ACTIVIDADES EN EL LABORATORIO¹

INTRODUCCIÓN

La tioacetamida es un compuesto orgánico blanco, cristalino, empleado inicialmente como fungicida en cultivos de naranjas. A partir del conocimiento de los efectos tóxicos de la tioacetamida se limitó su uso para cultivo y actualmente se utiliza en las industrias textiles, del cuero y papeleras (Sittig, 1985). Sin embargo, el principal uso que se le da en la actualidad es como modelo de toxina para el estudio de la hepatotoxicidad. También se emplea como modelo de hepatocarcinogenesis y cirrosis, ya que su administración crónica produce estas patologías (Zimmerman, 1999). La activación de la tioacetamida a su metabolito tóxico ocurre mediante dos oxidaciones cerradas, generando compuestos reactivos que inician la necrosis hepática al unirse covalentemente a diferentes macromoléculas. También se generan especies reactivas del oxígeno como productos secundarios de la oxidación microsomal de la tioacetamida, los cuales consumen el glutatión GSH generando estrés oxidativo en las células, contribuyendo con el daño hepático (Zimmerman, 1999).

En nuestro modelo de trabajo, la rata, una única administración de tioacetamida produce necrosis hepática, alcanzando entre las 24 y 48 horas el máximo daño, posteriormente el hígado comienza a regenerarse recuperándose casi completamente 7 días después. Los microARNs (miARNs) son moléculas de ARN pequeñas que regulan negativamente la expresión génica a nivel post-transcripcional, y están involucrados en numerosos procesos fisiológicos y fisiopatológicos. Está demostrado que el microARN-122 disminuye su expresión después de 24 hs en hígados de ratones tratados con tioacetamida, mientras que el mismo se ve aumentado en la sangre de dichos animales (Lardizabal et al, 2013). Se postula que este microARN podría emplearse como biomarcador de daño hepático.

El objetivo de las actividades prácticas del laboratorio del curso es determinar la expresión del microARN-122 en hígado y en sangre en un modelo de hepatotoxicidad aguda inducido por tioacetamida en la rata.

¹ La Guía de actividades de laboratorio fue confeccionada por María Noelia Lardizábal y Luis Veggi. Agradecemos los aportes y correcciones realizados por María Florencia Ércoli, y Carla Schommer

ACTIVIDADES PREVIAS REALIZADAS POR LOS DOCENTES DEL CURSO PARA LA OBTENCIÓN DE LAS MUESTRAS

TRATAMIENTO DE LOS ANIMALES²

GRUPO TRATADO: inyectar ratas (n=6) Wistar macho adultas (90 días) intraperitonealmente con 150 mg tioacetamida / kg de peso corporal (vehículo: solución fisiológica). Sacrificar a las 24 hs mediante pneumotórax.

GRUPO CONTROL: Inyectar ratas (n=6) Wistar macho adultas (90 días) intraperitonealmente con solución fisiológica 6 ml/kg de peso corporal y sacrificar a las 24 hs.

RECOLECCIÓN DE MUESTRAS DE HÍGADO Y SANGRE ENTERA DE RATA³

REACTIVOS

- TriPure Isolation Reagent (Roche; Nro. Cat: 11 667 157 001).

EQUIPOS

- Microcentrífuga.

MATERIAL NECESARIO POR EQUIPO DE TRABAJO

- Guantes.
- Gradilla de tubos eppendorf.
- Micropipetas de 20-200 ul; 200-1000 ul.
- Conservadora y hielo.
- Papel absorbente.
- Tubos eppendorf RNasa/DNasa free (Axygen MCT-150-C).

² Realizado por los docentes del curso.

³ Realizado por los docentes del curso.

- Tips estériles [Tips Amarillos (Axygen; Código: TR-222-Y), Tips Blancos (Axygen; Código: J T-300)].

RECOLECCIÓN MUESTRAS DE HÍGADO

1. Extraer el el lóbulo mayor del hígado y lavar con solución fisiológica.
2. Envolver en papel de aluminio y sumergirlo rápidamente en termo con N₂ (l).
3. Almacenar a -70°C.

RECOLECCIÓN DE MUESTRAS DE SANGRE

1. Preparar tubos eppendorf (RNasa free) con 20 ul de solución EDTA Anticoagulante (Wiener Lab). Dar leves golpecitos para salpicar las paredes del tubo.
2. Preparar tubos eppendorf (RNasa free) con 1 ml de TriPure.
3. Recolectar las muestras de sangre por punción cardíaca.
4. Llenar los tubos tratados con EDTA con aprox. 1 ml de sangre.
5. Homogenizar rápidamente.
6. Tomar 100 ul de sangre y llevarlos a los tubos con TriPure. Homogenizar.
7. Almacenar a -70°C hasta el día de extracción de RNA.

Día 1

PURIFICACIÓN Y CUANTIFICACIÓN DEL RNA

PURIFICACIÓN DE RNA

Se formarán dos comisiones. La comisión A trabajará con muestras de sangres, mientras que la comisión B empleará muestras de hígado. Cada comisión se dividirá en seis grupos, cada uno de ellos procesará una muestra proveniente del grupo control y una muestra proveniente del grupo tratado.

REACTIVOS

- Cloroformo (Merck; Nro. Cat: 1024452500).
- Etanol 100% (Merck; Nro. Cat: 1.00983.1000).
- H₂O libre de RNasa.
- PureLink RNA Mini Kit (Ambion; Nro. Cat: 12183018A).

EQUIPOS

- Centrífuga.
- Microcentrífuga.

MATERIAL NECESARIO POR EQUIPO DE TRABAJO

- Guantes.
- Gradilla de tubos eppendorf.
- Micropipetas de 2-20 ul; 20-200 ul; 200-1000 ul.
- Conservadora y hielo.
- Papel absorbente.
- Tubos eppendorf RNasa/DNasa free (Axygen MCT-150-C).
- Tips estériles [Tips Amarillos (Axygen; Código: TR-222-Y), Tips Blancos (Axygen; Código: J T-300)].

PROTOCOLO

1. Descongelar las muestras e incubar 5 min a temperatura ambiente.
2. Agregar 200 ul de cloroformo. Agitar vigorosamente por 15 seg (no vortexear).
3. Incubar 2 min a temperatura ambiente.

4. Centrifugar a $>12.000g$ (13.000 g) por 15 min.
5. Transferir la fase acuosa (si la muestra inicial es sangre ~ 500 ul y si es hígado ~ 400 ul) a un nuevo tubo (tubo estéril de plástico de 1.5 mL, no provisto en el kit).
6. Agregar un volumen equivalente de Etanol 100%.
7. Vortexear.
8. Si observa un precipitado en el tubo mezclar por inversión.
9. Transferir 700 ul a un *Spin Cartridge* (con *Collection Tube*), rotular el spin cartridge para identificar la muestra que se esta procesando.
10. Centrifugar a $>12.000g$ (13.000 g) por 1 min, a Ta.
11. Descartar el contenido del *Collection Tube*.
12. Transferir el contenido restante de la solución original al *Spin Cartridge* y repetir la centrifugación.
13. Descartar el *Collection Tube* , colocar el *Spin Cartridge* en un nuevo *Collection Tube*.
14. Agregar 500 ul de *Wash Buffer II* con etanol por el centro del *Spin Cartridge* (no por las paredes).
15. Centrifugar a $>12.000g$ (13.000 g) por 1min, a Ta.
16. Descartar el contenido del *Collection Tube*.
17. Agregar nuevamente 500 ul de *Wash Buffer II* con etanol.
18. Centrifugar a $>12.000g$ (13.000 g) por 1 min, a Ta.
19. Descartar el contenido del *Collection Tube*.
20. Centrifugar a $>12.000g$ (13.000 g) por 3 min, a Ta.
21. Descartar el *Collection Tube* , colocar el *Spin Cartridge* en el *Recovery Tube*.
22. Agregar 30 ul (muestras de sangre) o 60 ul (muestras de hígado) de *RNase-Free Water* en el *Spin Cartridge* por el centro (no por las paredes).
23. Incubar 1 min a Ta.
24. Centrifugar a $>12.000g$ (13.000 g) por 2 min, a Ta.
25. Almacenar a $-70^{\circ}C$.

CUANTIFICACIÓN DEL RNA OBTENIDO POR ESPECTROFOTOMETRÍA

MATERIALES

- Espectrofotómetro.
- H_2O libre de RNasa.
- Cubeta para luz UV.
- Papel absorbente.
- Tips/tubos.
- Pizeta.

ESPECTROFOTÓMETRO

Encender el equipo mínimamente 1/2 hora antes de su uso.
Realizar diluciones 1/100 de las muestras (8 ul en $V_f=800$ ul).

SETEAR EN EL EQUIPO:

- Wavelength 1: 260 nm.
- Dilution Factor: 100.
- Units: ng/ul.
- Factor: 40.
- Lámpara de deuterio.

Registrar los valores de Concentración (A_{260}).
Lavar la cubeta con abundante agua, escurrir.
Antes del tratamiento con DNAsa si la concentración de RNA obtenida es superior a ~ 400 ng/ul, previamente diluir las muestras a una concentración aprox de 200 ng/ul, y cuantificar nuevamente.

Previa Día 2

CHEQUEO DE LA INTEGRIDAD DEL RNA

REACTIVOS

- Tris Base.
- Acido Acético glacial.
- EDTA.
- NaOH 0.5 M.
- Agarosa.
- EtBr.
- Loading Buffer.
- H₂O estéril.
- RNA para control positivo de integridad en la electroforesis.

EQUIPOS

- Autoclave.
- pHímetro.
- Microondas.
- Cuba electroforética.
- Microcentrífuga.
- Baño a 65°C.

PREPARACIÓN SOLUCIÓN: TAE 50X (V_F = 250 mL)

- | | |
|-------------------------|---------|
| • Tris base | 60.5 g |
| • Acido acético glacial | 14.3 ml |
| • 0.5 M EDTA (pH 8.0) | 25 ml |

PREPARACIÓN SOLUCIÓN: EDTA 0.5 M (V_F = 100 mL)

Agregar 18.6 g a 80 mL de agua. Agitar vigorosamente, ajustando a pH 8.0 con NaOH (aprox. 2g de perlitas). El EDTA no se disuelve hasta que el pH llega a 8.0. Esterilizar por autoclave.

PROCEDIMIENTO

1. Tratar la cuba de electroforesis 1 hora aprox. con NaOH 0,5 M.
2. Enjuagar con abundante agua estéril.
3. Preparar 500 ml de TAE 1X con agua estéril.
4. Pesar 0.75 g de agarosa y disolver en 50 ml de TAE en erlenmeyer (Agarosa: 1.5 %)
5. Calentar en microondas hasta que se disuelva la agarosa (debe quedar completamente disuelta)
6. Dejar enfriar hasta ~ 60°C
7. Agregar 8 ul de EtBr
8. Armar la cuba con el peine sobre el contenedor.
9. Volcar la agarosa en la cuba cuidando que no se formen burbujas. Dejar que gelifique (aprox 20 min)
10. Preparación de las muestras: diluir el RNA en H₂O DEPC de manera de tener ~ 2 ug finales para el caso de RNA extraído de tejido hepático, y 0.6 ug para RNA obtenido de sangre, para nuestro caso 4 ul de ARN de hígado y 8 ul ARN de sangre es adecuado.
11. Incluir un control positivo: una muestra cuya integridad ya fue probada
12. Sembrar las muestras con 3 ul de Loading Buffer
13. Correr el gel a 100 V por ~ 15 min
14. Observar el gel en transiluminador de luz UV, scanear la imagen
15. Cuantificar las bandas por densitometría, calcular el índice 28S/18S

Día 2

SÍNTESIS DEL cDNA

SE DIVIDIRÁ LA COMISIÓN EN CUATRO GRUPOS CON DIFERENTES TAREAS:

- Síntesis de cDNA para cuatro ARN controles y cuatro tratados incluyendo tratamiento con DNAasa (grupo de cuatro estudiantes).
- Síntesis de cDNA por duplicados en una muestra control incluyendo tratamiento con DNAasa (grupo de tres estudiantes).
- Síntesis de cDNA por duplicados en una muestra tratada incluyendo tratamiento con DNAasa (grupo de tres estudiantes).
- Reacción control de verificación de actividad de DNAasa (grupo de dos estudiantes).

REACTIVOS

- DNasa (Promega; Nro. Cat: M610A).
- 10X Buffer RQ1 DNasa (Kit DNasa, Promega).
- DNasa Stop Solution (Kit DNasa, Promega).
- H₂O miliQ.
- Oligo(dTV).
- dNTPs (GeneAmp dNTP Mix with dTTP; Applied Biosystem; Nro. Cat: N8080260).
- MMLV (Invitrogen; Nro. Cat: 28025-013).
- 5X First-Strand Buffer (Kit MMLV, Invitrogen).
- DTT 0.1 M (Kit MMLV, Invitrogen).
- Inhibidor de RNasa (RNaseOUT; Invitrogen; Nro. Cat: 10777-019).

EQUIPOS

- Baño termostatzado 37 C en baño seco y 60 C en baño de agua.
- Termociclador.

MATERIAL NECESARIO POR EQUIPO DE TRABAJO

- Gradilla de tubos eppendorf.
- Micropipetas de 2-20 ul; 20-200 ul; 200-1000 ul.
- Conservadora y hielo.
- Papel absorbente.
- Tubos eppendorf RNasa/DNasa free (Axygen MCT-150-C).
- Tips estériles [Tips Amarillos (Axygen; Código: TR-222-Y), Tips Blancos (Axygen; Código: J T-300)].

- Microtubos para PCR (0.2ml Thin Wall Tubes 8 Per Strip. Axygen; Código: PCR-0208-C)
- Micropipeta 0-2 ul

I) TRATAMIENTO CON DNAsa

Utilizar las muestras de acuerdo a la integridad verificada en las imágenes de las corridas electroforéticas en los geles de agarosa.

PREPARAR LA SIGUIENTE REACCIÓN:

ARN	1.2 UG (PIPETEAR 5UL MINIMO)
10X BUFFER RQ1 DNAsa	1 UL
DNAsa	1 UL
H ₂ O MILIQ	CSP 10 UL

1. Incubar 30 minutos a 37 °C
2. Agregar 1 ul de RQ1 DNasa Stop Solution
3. Inactivación: 10 minutos a 65 °C, realizar un spin

De este tubo de reacción se usarán 8 uL para armar la reacción de retro-transcripción. Con lo restante de cada tubo (3 ul aprox) se realizará un pool, el cual será el tubo de control de tratamiento con DNAsa. Éste se procesa igual que los demás, excepto que no se agrega la retrotranscriptasa.

2) STEM LOOP-RT

SLO: *Stem Loop Oligo*

PREPARAR LA SIGUIENTE MIX:

Mix I: *oligo-dTV + dNTPs mix + mix de oligos*

REACTIVO	Cc INICIAL	VOL	Cc FINAL
OLIGO(dTV)	0,5 uG/UL *	1 UL	0.025 uG/UL
dNTPs	10 mM	1 UL	0.5 mM
MIX OLIGOS	0.1 uM SLO; 25 uM 18S RT; 7.5 uM 5S RT	2 UL	10 nM SLO; 2.5 uM 18S RT; 0.75 uM 5S RT

* la solución stock es 2 ug/ul (Invitrogen)

MIX DE OLIGOS (PARA $V_F = 10 \text{ UL}$)⁴:

- 0,1 ul SLO (10 uM) → de cada uno de los SLO
- 2,5 ul 18S RT (100 uM)
- 0.75 ul 5S RT (100 uM)

1. Agregar a la Mix 1 de las muestras de ARN 8 ul por tubo ($V_f = 12 \text{ ul}$).
2. Vortear. Spin down.
3. Calentar a 65 °C por 5 minutos.
4. Incubar en hielo al menos 1 minuto (rápido).
5. Spin down.

PREPARAR LA SIGUIENTE MIX:

Mix II: Buffer + DTT + RNase OUT

REACTIVO	Cc INICIAL	VOL	Cc FINAL
5X FIRST-STRAND BUFFER	5X	4 UL	1X
DTT	0.1 M	2 UL	10 MM
INHIBIDOR DE RNASA (RNASEOUT)	40 U/UL	1 UL	2 U/UL
MMLV	200 U/UL	1 UL	10 U/UL

Mix III: Buffer + DTT + H_2O (Para el control negativo)

REACTIVO	Cc INICIAL	VOL	Cc FINAL
5X FIRST-STRAND BUFFER	5X	4 UL	1X
DTT	0.1 M	2 UL	10 MM
H_2O	-	2 UL	-

1. Agregar la Mix 2 o la Mix 3 según el caso al tubo que tiene la Mix 1 mas el ARN.
2. Vortear. Spin down.
3. Correr el siguiente programa en termociclador:

TIEMPO (MIN)	TEMPERATURA (°C)
30	16
50	37
15	70
1	25

4. Almacenar a -20 °C (sin diluir)

⁴ En nuestro caso provista por los docentes.

Día 3

qPCR

SE DIVIDIRÁ LA COMISIÓN EN GRUPOS CON DIFERENTE TAREAS:

- GRUPO 1 qPCR miR-122 de las 4 muestras controles y las cuatro muestras tratadas por duplicado.
- GRUPO 2 qPCR miR-16 de las 4 muestras controles y las cuatro muestras tratadas por duplicado.
- GRUPO 3 qPCR miR-let7a de las 4 muestras controles y las cuatro muestras tratadas por duplicado.
- GRUPO 4 qPCR 18S (comisión muestras de sangre) o 5S (comisión muestras de hígado) de las 4 muestras controles y las cuatro muestras tratadas por duplicado.
- GRUPO 5 qPCR control (+) y NTC miR122, miR16, 5S y let7a.
- GRUPO 6 qPCR miR16 en los duplicados RT de las muestra control y de la tratada por duplicado y rea lizar muestras control de actividad ADNAsas.

REACTIVOS

- Platinum® Taq DNA Polymerase (Invitrogen; Cat. Nro: 10966-018)
- 10X PCR Buffer –MgCl₂ (Kit Taq Platinum).
- 50 mM MgCl₂ (Kit Taq Platinum)
- dNTPs (GeneAmp dNTP Mix with dTTP; Applied Biosystem; Nro. Cat: N8080260)
- SYBR Green I (Invitrogen; Cat. Nro: S7567)

EQUIPOS

- Equipo de Real Time PCR: Termociclador Mx3000P (Stratagene).

MATERIAL NECESARIO POR EQUIPO DE TRABAJO

- Gradilla de tubos eppendorf.
- Micropipetas de 2-20 ul; 20-200 ul; 200-1000 ul.
- Papel absorbente, conservadora y hielo.
- Tubos eppendorf RNasa/DNasa free (Axygen MCT-150-C).
- Tips estériles [Tips Amarillos (Axygen; Código: TR-222-Y), Tips Blancos (Axygen; Código: J T-300)].
- Micropipeta 0-2 ul.
- Tubos Eppendorf 1,5 mL (Axygen; Código: MCT-150-C).

- Microtubos para PCR (0.2ml Thin Wall Tubes 8 Per Strip. Axygen; Código: PCR-0208-C).
- Tapas para strips de 8 tubos (flat top) (Axygen; Código: PCR-2CP-RT-C).

PREPARACIÓN DE LOS TUBOS PARA LA REALTIME PCR

CON FASTSTART UNIVERSAL SYBR GREEN MASTER (ROX) VERSION 3 (Roche; NRO. CAT: 04913850001) o SYBR® SELECT MASTER MIX (APPLIED BIOSYSTEMS; NRO. CAT: 4472903)

1. Diluir los cDNA 1/40 (en H₂O mQ) considerando: a) que el volumen inicial a pipetear sea mayor o igual a 4 ul; b) el volumen necesario de cDNA para todas las determinaciones.
2. Preparar en el momento una mix de primers por cada gen a determinar (forward y reverse): para 100 ul → 2.5 ul Forward (100 uM) + 2.5 ul Reverse (100 uM) + 95 ul H2O mQ → Cf: 2.5 uM de cada primer.
3. Agregar por cada tubo: 10 ul Mix comercial + 4 ul mix de oligos 2.5 uM (oligos forward y reverse) + 6 ul cDNA.
4. Incluir control negativo (NTC: No Template Control) por cada gen a determinar: contiene mix + primers + H2O mQ.
5. Incluir control positivo por cada gen a determinar: contiene mix + primers + cDNA Standard.
6. Spin down.
7. Correr el siguiente programa en equipo de Real Time PCR:

PROGRAMA KIT SYBR® SELECT MASTER MIX (APPLIED BIOSYSTEMS; NRO. CAT: 4472903)

ETAPA	T (°C)	TIEMPO	NRO DE CICLOS
1	50	2 MIN	1
2	95	2 MIN	1
3	95	15 SEG	40
4	60 (PUNTO DE LECTURA)	60 SEG	
5	CURVA DE MELTING (VIENE POR DEFAULT EN EL PROGRAMA)		

PROGRAMA KIT FASTSTART UNIVERSAL SYBR GREEN MASTER (ROX) VERSION 3 (ROCHE; NRO. CAT: 04913850001)

ETAPA	T (°C)	TIEMPO	NRO DE CICLOS
1	95	10 MIN	1
2	95	15 SEG	40
3	60	30 SEG	
4	72 (PUNTO DE LECTURA)	40 SEG	
5	CURVA DE MELTING (VIENE POR DEFAULT EN EL PROGRAMA)		

EQUIPO DE REAL TIME PCR

PARA EMPEZAR LA CORRIDA

1. Abrir programa (MxPro, se encuentra en el escritorio).
2. New Options - SYBR with dissociation curve → OK
3. Pestaña “Thermal profile set up” → setear el programa de PCR
4. Arrastrar la lupa “end” al punto donde se desea medir la fluorescencia
5. Seleccionar “Plate set up”
6. Seleccionar los wells donde van los tubos
7. A la derecha, seleccionar “well type” y luego “unknown”. Seleccionar SYBR en el panel de abajo
8. Cuando se usa FastStart Universal SYBR Green Master (ROX) (Roche): seleccionar ROX en el menú Reference Dye.
9. Para dar nombre a los wells si se desea (no es imprescindible): en el panel de la derecha clicar “Assign Assay Names”. Se abre una nueva ventana, seleccionar SYBR y debajo de “Assay” tipear el nombre. Destildar la opción “Use for all wells”
10. Clicar “Run” (arriba a la derecha)
11. Dar “Start”. (IMPORTANTE: Seleccionar la opción de apagar la lámpara al finalizar la corrida).
12. Dar nombre al archivo y guardarlo
13. Elegir la opción “Run after warm-up”.

PARA ANALIZAR LOS RESULTADOS DURANTE LA CORRIDA

1. Seleccionar la pestaña “Analysis”. Los datos se ven en una nueva ventana (en la pestaña “Results”), mientras la otra sigue corriendo.
2. En el panel de la derecha se puede elegir ver “Amplification plots”, “Dissociation curves”, etc.
3. El threshold (que se setea automáticamente) se puede cambiar moviéndolo desde el gráfico.

4. Para ver el gráfico de amplificación en escala logarítmica: clicar botón derecho sobre el eje de fluorescencia – seleccionar “Scale Y axis” y luego “Log”.

PARA ANALIZAR LOS RESULTADOS AL FINAL DE LA CORRIDA

1. En el panel de la derecha, tildar la opción “Text report”. Seleccionar los datos.
2. Clicar botón derecho: “File”- “Export text report”-“Export text report to excel”
3. Seguir las instrucciones sobre el uso seguro de pendrives al sacar sus datos.

Día 4

ANÁLISIS DE RESULTADOS

CONTROLES DE TRASCRIPTIÓN REVERSA

1. Control de DNasa, qPCR con pool de muestras controles.
2. Control de síntesis, qPCR de pool para control positivo.

CONTROLES DE qPCR

1. NTC (*no template control*), uno por cada gen en estudio, se pueden observar amplificaciones por contaminación o por dímeros de cebadores (*primer dimer*). Estos dos se diferencian observando la curva de melting. La contaminación es aceptable cuando existe una diferencia de al menos 8 ciclos respecto al Cq del GOI (gen de interés) en las muestras y cuando el blanco amplifica entre los ciclos 35 a 40. Se realiza un gel de agarosa 2 % con bromuro de etidio para confirmar dímero de cebadores de contaminación. Comparar en caso se observe amplificación la curva de melting de NTC con la del control positivo.
2. Control de especificidad curvas de melting, opcional realizar gel de agarosa 2% con bromuro de etidio.
3. Control de línea de cuantificación para Cq y línea de base, en caso de la primera ver si se corren diferentes genes juntos en una misma corrida que la línea de cuantificación atraviese sobre la fase exponencial de los perfiles de amplificación por cada gen en estudio (GOI o RG). En general ambos son determinados automáticamente por el software del equipo.
4. Control positivo, uno por cada gen en estudio, controlar valor Cq respecto a valores históricos. Controlar semi cuantitativamente la eficiencia sobre curvas de amplificación (mirando las pendientes de la amplificación en escala logarítmica).
5. Repetibilidad: La desviación estándar de la replicas técnicas de qPCR (duplicados y triplicados) no mayor a 0.3, con un promedio de 0.1 (problemas de eficiencia o problemas de pipeteo). Evaluar la desviación estándar de la replicas técnicas de RT (duplicados y triplicados), (problemas de eficiencia o problemas de pipeteo). Esta réplica prácticamente no se realiza en general, sin embargo la variabilidad de la reacción de RT es mucho mayor que la de qPCR.
6. Control Normalización: Análisis de estabilidad entre genes de referencia, se calcula los parámetros M (variación promedio evaluada analizando los genes de a pares) y el coeficiente de variación (CV) de los cantidades relativas normalizadas de los genes de referencia. Los valores aceptables son menores a 0.5 y 25 % respectivamente.

Procesamiento analítico de los Cq para calcular las cantidades relativas normalizadas de expresión de cada GOI (pasos, promedio Cq

replica técnicas, calculo cantidades relativas, calculo cantidades relativas normalizadas).

ANÁLISIS ESTADÍSTICOS DE LAS VARIACIONES DE EXPRESIÓN ENTRE LOS GRUPOS EXPERIMENTALES EN ESTUDIO.

1. Transformación logarítmica.
2. Tests estadísticos.

Anexo 1

INFORMACIÓN DE CONTACTO DE LOS DOCENTES DEL CURSO

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Anexo 2

**DISTRIBUCIÓN DE TAREAS
PARA CADA COMISIÓN
DISCRIMINANDO POR
GRUPO Y POR DÍA**

Anexo 3

**INSTRUCTIVO CORTO PARA
LA REALIZACIÓN DE
TRATAMIENTO CON DNAsa,
RT Y qPCR DISCRIMINADO
POR GRUPO**

Tratamiento con DNasa

$$\text{cc (ng/ul)} = A_{260} * 40 * \text{Factor Dil}$$

$$\text{DIL } 1/100$$

		Tratamiento DNasa					
	Muestra	Rata	A260	Cc (ng/ul), AB260*4000	Vol RNA (ul) que contiene 1200 ng	Vol H2O (ul) para completar 8 uL	
Grupo 1		C1					Mix Dnasa grupo 1 9 ul Buffer RQ1 DNasa + 9 ul DNasa Alicuotar 2 ul por tubo
		C2					
		C2					
		C4					
		AT1					
		AT2					
		AT3					
		AT4					
Gr 2							Mix Dnasa grupo 2 3 ul Buffer RQ1 DNasa + 3 ul DNasa Alicuotar 2 ul por tubo
		C1					
		C1					
Gr 3							Mix Dnasa grupo 3 3 ul Buffer RQ1 DNasa + 3 ul DNasa Alicuotar 2 ul por tubo
		AT1					
		AT1					

Incubar 30 min a 37 °C
agregar 1 ul de STOP Solution
Inactivación: 10 minutos a 65 °C

RT

	Muestra	Rata		MIX grupo 1	Vol (ul)	
Grupo 1		C1	cada tuno agregar 8 ul del tubo de tratamiento con DNAsa	Oligo(dT) 0.5 ug/ul	10	Alicuotar 4 ul/tubo
		C2		dNTP 10 mM	10	
		C2		Mix Oligos	20	
		C4				
		AT1				
		AT2				
		AT3				
		AT4				
				MIX grupo 2	Vol (ul)	
Gr 2		C1	cada tuno agregar 8 ul del tubo de tratamiento con DNAsa	Oligo(dT) 0.5 ug/ul	2,5	Alicuotar 4 ul/tubo
		C1		dNTP 10 mM	2,5	
				Mix Oligos	5	
Gr 3		AT1	cada tuno agregar 8 ul del tubo de tratamiento con DNAsa	MIX grupo 3	Vol (ul)	Alicuotar 4 ul/tubo
		AT1		Oligo(dT) 0.5 ug/ul	2,5	
				dNTP 10 mM	2,5	
				Mix Oligos	5	
Gr 4		cont act DNAsa 1	en cada tubo agregar 1 ul del tubo de tratamiento con DNAsa de 6 muestras diferentes y completar a 8ul con H2O dest	MIX grupo 4	Vol (ul)	Alicuotar 4 ul/tubo
		cont act DNAsa 2		Oligo(dT) 0.5 ug/ul	2,5	
				dNTP 10 mM	2,5	
				Mix Oligos	5	

Para los grupos 1,2, 3 y 4
Vorterear. Spin down
Calentar a 65°C por 5 min (baño seco)
Incubar rápido en hielo al menos 1 min
Spin down

GRUPO 1: síntesis cDNA muestras control y tratadas

MIX II	Vol (ul)
1X First-Strand Buff	36
DTT 0.1 M	18
RNase Out	9
MMLV	9

GRUPO 2: Síntesis de cDNA a partir de control por duplicado

GRUPO 3: Síntesis de cDNA a partir de tratada por duplicado

GRUPO 4: control activ DNAsa

MIX II	Vol (ul)
5X First-Strand Buffer	8,8
DTT 0.1 M	4,4
RNase Out	2,2
MMLV	2,2

grupo 4 poner agua

Para los grupos 1,2, 3 y 4

Agregar 7 ul de esta mix a los tubos anteriores
Vorterear. Spin down
Trasvasar a los tubos para PCR

qPCR

un grupo entre el 1,2, 3 o 4 diluye los cDNA de las 4 muestra tratadas y de las 4 muestras controles
diluir cDNA 1 en 40
en tubos 1,5 ml estériles
10 ul cDNA más 390 uL de agua

grupo 1 qPCR miR-122 de las 4 muestras controles y las cuatro muestras tratadas por duplicado
por cada tubo poner

grupo 2 qPCR miR-16 de las 4 muestras controles y las cuatro muestras tratadas por duplicado

grupo 3 qPCR miR-let7a de las 4 muestras controles y las cuatro muestras tratadas por duplicado

grupo 4 qPCR 18S (comisión muestras de sangre) o 5S (comisión muestras de hígado)

de las 4 muestras controles y las cuatro muestras tratadas por duplicado

10	ul mix comercial
4	ul mix de oligos
6	ul cDNA dil 1/40

grupo 5 qPCR con pos y neg miR122, miR16, let7a y 5S o 18S segun si es comision 1 o 2

10	ul mix comercial
4	ul mix de oligos
6	ul cDNA control positivo o agua

grupo 6 qPCR miR16 en los duplicados RT de las muestra control y de la tratada por duplicado
y realizar muestras control de actividad ADNAsas

realizar las diluciones 1 en 40 de los cDNA de las muestras realizadas por duplicados
diluir cDNA 1 en 40
en tubos 1,5 ml estériles
10 ul cDNA más 390 uL de agua

10	ul mix comercial
4	ul mix de oligos
6	ul cDNA dil 1/40

Anexo 4

PROTOCOLOS ADICIONALES

PURIFICACIÓN CON TriPURE O TRIZOL

REACTIVOS

- Cloroformo (Merck; Nro. Cat: 1024452500)
- Isopropanol (Merck; Nro. Cat: 1.09634.1011)
- Etanol 75% (Merck; Nro. Cat: 1.00983.1000)
- GlycoBlue (Ambion; Nro. Cat: 9515)
- H₂O libre de RNasa
- PureLink RNA Mini Kit (Ambion; Nro. Cat: 12183018A)

EQUIPOS

- Centrífuga refrigerada
- Baño seco
- Microcentrífuga
- Estufa

PROTOCOLO

1. Descongelar las muestras e incubar 5 min a temperatura ambiente.
2. Agregar 200 ul de cloroformo. Agitar 15 seg (no vortexear)
3. Incubar 2 min a temperatura ambiente.
4. Centrifugar a 12.000 g por 15 min, entre 2 y 8 °C.
5. Transferir la fase acuosa a un nuevo tubo.
6. Agregar 2 ul de GlycoBlue
7. Precipitar el RNA con 500 ul de isopropanol frío
8. Mezclar por inversión. Incubar 5 – 10 min a temperatura ambiente (u ON a -20°C).
9. Centrifugar a 12.000 g 10 min a 2 – 8 °C y descartar el SN.
10. Lavar con 1 mL de etanol 75% frío.
11. Centrifugar a 7500 g, 5 min a 2 – 8°C.
12. Descartar el SN.
13. Secar el pellet 5 min a 37° (estufa). No dejar secar el pellet completamente ya que luego será imposible resuspenderlo.
14. Resuspender en 20 ul de agua miliQ, libre de RNasa.
15. Incubar 10-15 min a 55-60°C

16. Centrifugar a 6500 rpm por 5 min. Trasvasar y fraccionar en tubos nuevos.
17. Almacenar a -70 °C.

PROTOCOLO qPCR CON TAQ PLATINUM / SYBR GREEN I (INVITROGEN)

1. Diluir los cDNA 1/40 (en H₂O mQ) considerando: a) que el volumen inicial a pipetear sea mayor o igual a 4 ul; b) el volumen necesario para todas las determinaciones.
2. Preparar en el momento una mix de primers por cada gen a determinar (forward y reverse): para 100 ul 2.5 ul Forward (100 uM) + 2.5 ul Reverse (100 uM) + 90 ul H₂O mQ Cf: 2.5 uM de cada primer
3. Preparar una mix de todos los reactivos excepto el cDNA y los primers.

REACTIVO	Cc INICIAL	VOLUMEN	Cc FINAL
H ₂ O	-	6,5 UL	-
10X PCR BUFFER –MgCl ₂	10X	2 UL	1X
MgCl ₂	50 mM	1,2 UL	3 mM
DNTPs	10 mM	0,4 UL	0,2 mM
SYBR GREEN I	10X	0,8 UL	0,4 X
PLATINUM® TAQ DNA POLYMERASE	5 U/UL	0,1 UL	0,025 U/UL

IMPORTANTE (SYBR Green I): la solución stock (10.000X) se diluye 1/10 en DMSO y esta se conserva a -20°C envuelto en papel aluminio. El día de la PCR se hace una dilución 1/100 en H₂O mQ, y se usa en el mismo día, no usar en días posteriores porque no se conserva correctamente.

1. Alicuotar la mix en los tubos Axygen (11 ul cada tubo).
2. Agregar sobre la pared del tubo 4 ul de mix de oligos 2.5 uM (oligos forward y reverse) Cf: 0.5 uM
3. Agregar cuidadosamente 5 ul de cDNA sobre la pared del tubo, verificando de emitir todo el volumen.
4. Incluir control negativo (NTC: *No Template Control*) por cada gen a determinar: contiene mix + primers + H₂O mQ
5. Incluir control positivo por cada gen a determinar: contiene mix + primers + cDNA Standard
6. Spin down
7. Correr el siguiente programa en equipo de Real Time PCR:

ETAPA	T (°C)	TIEMPO	NRO DE CICLOS
1	95	1 MIN	1
2	95	15 SEG	40
3	60	30 SEG	
4	72 (PUNTO DE LECTURA)	40 SEG	
5	CURVA DE MELTING (VIENE POR DEFAULT EN EL PROGRAMA)		

Anexo 5

TRABAJO ORIGINAL DE MIQE

The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

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BACKGROUND: Currently, a lack of consensus exists on how best to perform and interpret quantitative real-time PCR (qPCR) experiments. The problem is exacerbated by a lack of sufficient experimental detail in many publications, which impedes a reader's ability to evaluate critically the quality of the results presented or to repeat the experiments.

CONTENT: The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. MIQE is a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments. Included is a checklist to accompany the initial submission of a manuscript to the publisher. By providing all relevant experimental conditions and assay characteristics, reviewers can assess the validity of the protocols used. Full disclosure of all reagents, sequences, and analysis methods is necessary to enable other investigators to reproduce results. MIQE details should be published either in abbreviated form or as an online supplement.

SUMMARY: Following these guidelines will encourage better experimental practice, allowing more reliable and unequivocal interpretation of qPCR results.

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The fluorescence-based quantitative real-time PCR (qPCR)¹⁵ (1–3), with its capacity to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources, is the enabling technology par excellence of molecular diagnostics, life sciences, agriculture, and medicine (4, 5). Its conceptual and practical simplicity, together with its combination of speed, sensitivity, and specificity in a homogeneous assay, have made it the touchstone for nucleic acid quantification. In addition to its use as a research tool, many diagnostic applications have been developed, including microbial quantification, gene dosage determination, identification of transgenes in genetically modified foods, risk assessment of cancer recurrence, and applications for forensic use (6–11).

This popularity is reflected in the prodigious number of publications reporting qPCR data, which invariably use diverse reagents, protocols, analysis methods, and reporting formats. This remarkable lack of consensus on how best to perform qPCR experiments has the adverse consequence of perpetuating a string of serious shortcomings that encumber its status as an independent yardstick (12). Technical deficiencies that affect assay performance include the following: (a) inadequate sample storage, preparation, and nucleic acid quality, yielding highly variable results; (b) poor choice of reverse-transcription primers and primers and probes for the PCR, leading to inefficient and less-than-robust assay performance;

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Received October 20, 2008; accepted January 27, 2009.

Previously published online at DOI: 10.1373/clinchem.2008.112797

¹⁵ Nonstandard abbreviations: qPCR, quantitative real-time PCR; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; RT-qPCR, reverse transcription-qPCR; FRET, fluorescence resonance energy transfer; C_q, quantification cycle, previously known as the threshold cycle (C_t), crossing point (C_p), or take-off point (TOP); RDML, Real-Time PCR Data Markup Language; LOD, limit of detection; NTC, no-template control.

and (c) inappropriate data and statistical analyses, generating results that can be highly misleading. Consequently, there is the real danger of the scientific literature being corrupted with a multitude of publications reporting inadequate and conflicting results (13). The publication (14) and retraction (15) of a *Science* “Breakthrough of the Year 2005” report provides a disquieting warning. The problem is exacerbated by the lack of information that characterizes most reports of studies that have used this technology, with many publications not providing sufficient experimental detail to permit the reader to critically evaluate the quality of the results presented or to repeat the experiments. Specifically, information about sample acquisition and handling, RNA quality and integrity, reverse-transcription details, PCR efficiencies, and analysis parameters are frequently omitted, whereas sample normalization is habitually carried out against single reference genes without adequate justification.

The aim of this document is to provide authors, reviewers, and editors specifications for the minimum information, set out in Table 1, that must be reported for a qPCR experiment to ensure its relevance, accuracy, correct interpretation, and repeatability. MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments, pronounced *mykee*) is modeled on similar guidelines drawn up for DNA microarray analysis (16), proteomics experiments (17), genome sequence specification (18), and those under discussion for RNA interference work (19, 20) and metabolomics (21), all of which are initiatives coordinated under the umbrella of MIBBI (Minimum Information for Biological and Biomedical Investigations, <http://www.mibbi.org>) (22). Compulsory inclusion of a common reporting language to allow data sharing is not proposed, although it is envisaged that a future update of these guidelines could include such a recommendation. Rather, these guidelines target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. They should be read in conjunction with recent publications that deal in depth with the issue of qPCR standardization (23–26).

1. Nomenclature

A few terms require standardization to ensure clarification:

- 1.1 We propose that the abbreviation qPCR be used for quantitative real-time PCR and that RT-qPCR be used for reverse transcription–qPCR. Applying the abbreviation RT-PCR to qPCR causes confu-

sion and is inconsistent with its use for conventional (legacy) reverse transcription–PCR.

- 1.2 Genes used for normalization should be referred to as *reference genes*, not as *housekeeping genes*.
- 1.3 TaqMan probes should be referred to as *hydrolysis probes*.
- 1.4 The term *FRET probe* (fluorescence resonance energy transfer probe) refers to a generic mechanism in which emission/quenching relies on the interaction between the electron-excitation states of 2 fluorescent dye molecules. LightCycler-type probes should be referred to as *dual hybridization probes*.
- 1.5 The *Oxford English Dictionary* lists only *quantification*, not *quantitation*; therefore, the former is the proper word.
- 1.6 The nomenclature describing the fractional PCR cycle used for quantification is inconsistent, with *threshold cycle* (C_t), *crossing point* (C_p), and *take-off point* (TOP) currently used in the literature. These terms all refer to the same value from the real-time instrument and were coined by competing manufacturers of real-time instruments for reasons of product differentiation, not scientific accuracy or clarity. We propose the use of *quantification cycle* (C_q), according to the RDML (Real-Time PCR Data Markup Language) data standard (<http://www.rdml.org>) (27).

2. Conceptual Considerations

To explain and justify the guidelines, we find it useful to review a number of key issues surrounding qPCR experiments:

- 2.1 *Analytical sensitivity* refers to the minimum number of copies in a sample that can be measured accurately with an assay, whereas *clinical sensitivity* is the percentage of individuals with a given disorder whom the assay identifies as positive for that condition. Typically, sensitivity is expressed as the *limit of detection* (LOD), which is the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure. The most sensitive LOD theoretically possible is 3 copies per PCR (28), assuming a Poisson distribution, a 95% chance of including at least 1 copy in the PCR, and single-copy detection. Experimental procedures typically include sample-processing steps (i.e., extraction) and, when required, reverse transcription. If the volume changes and the efficiencies of these steps are accounted for, the most sensitive LOD theoretically possible can be expressed in units relevant to the experiment, such as copies per nanogram of tissue. Experimental results less than the theoretic-

Table 1. MIQE checklist for authors, reviewers, and editors.^a

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D ^d
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg ²⁺ , and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE ^b samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A ₂₆₀ /A ₂₈₀)	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C _q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and y intercept	E
RIN/RQI or C _q of 3' and 5' transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D
Inhibition testing (C _q dilutions, spike, or other)	E	r ² of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C _q variation at LOD	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C _q s with and without reverse transcription	D ^c	Method of C _q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C _q or raw data submission with RDML	D

^a All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.

^b FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.

^c Assessing the absence of DNA with a no–reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no–reverse transcription control is desirable but no longer essential.

^d Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

cally possible LOD should never be reported. It also follows that results of “0” are meaningless and misleading. LOD estimates in qPCR analyses are complicated by the logarithmic nature of C_q , because C_q is undefined when the template concentration is zero. Appropriate determination and modeling of the LOD in the qPCR is the focus of continued research (26).

- 2.2 *Analytical specificity* refers to the qPCR assay detecting the appropriate target sequence rather than other, nonspecific targets also present in a sample. *Diagnostic specificity* is the percentage of individuals without a given condition whom the assay identifies as negative for that condition.
- 2.3 *Accuracy* refers to the difference between experimentally measured and actual concentrations, presented as fold changes or copy number estimates.
- 2.4 *Repeatability* (short-term precision or intraassay variance) refers to the precision and robustness of the assay with the same samples repeatedly analyzed in the same assay. It may be expressed as the SD for the C_q variance. Alternatively, the SD or the CV for copy number or concentration variance may be used. CVs should not be used with C_q s, however (29).
- 2.5 *Reproducibility* (long-term precision or interassay variance) refers to the variation in results between runs or between different laboratories and is typically expressed as the SD or CV of copy numbers or concentrations. C_q values generated from different runs are subject to inherent interrune variation (30); hence, reporting interrune C_q variation is not appropriate.

Publications describing mRNA concentrations for target genes should make it clear precisely what the targets are. The transcripts of most human genes and many genes in other multicellular organisms are alternatively spliced (31, 32), and these splicing variants specify alternative protein isoforms, with variation in splicing patterns reported in different tissues or at different developmental stages. Consequently, single exon-based RT-qPCR assays may detect a number of splice variants, whereas intron-spanning primers may be more selective but may miss some splice variants altogether. Most recently, autosomal nonimprinted genes that display allelic imbalance in their expression have been described (33). Taken together, these findings imply that use of an RT-qPCR assay that simply targets one or at most 2 exons of an mRNA is no longer sufficient to describe the expression level of a particular gene. Consequently, sequence information for primers must be provided together with an assessment of their specificity with respect to known splice variants and

single-nucleotide polymorphism positions documented in transcript and single-nucleotide polymorphism databases. For primer sets selected from the RTprimerDB database (34, 35), this is easily done by consulting the RTprimerDB Web site (<http://www.rtpimerdb.org>), which contains all the relevant information. For commercial assays, lot information and the providers' experimental validation criteria are required. The reporting of results for nonvalidated commercial assays and assays that have been validated only in silico are strongly discouraged.

It must be remembered that detection of the presence of an mRNA provides no information on whether that mRNA will be translated into a protein or, indeed, whether a functional protein is translated at all.

Immunohistochemistry, western blotting, or other protein-quantification methods are not always able to corroborate quantitative cellular mRNA data. It is now well established that there is frequently a lack of concordance between mRNA- and protein-concentration data (36), which is particularly true for mRNAs that specify proteins that are part of multifunction protein complexes (37). Finally, it has become clear that knowledge of the presence and function of specific microRNAs is as important to understanding gene expression as being able to quantify the mRNA species (38).

It is also necessary to be aware that most quantitative RNA data are not absolute, but relative. Thus, the reference genes or materials used for standardization are critical, and any assessment of the validity of an RT-qPCR experiment must also consider the appropriateness of the relative-quantification reference. Therefore, the development of universal reference DNA and RNA calibration materials, although very helpful (39, 40), will not be a universal panacea (41, 42).

Much of the variance in reported expression values produced in RT-qPCR experiments is not simply due to variation in experimental protocols but is caused by corrections applied by various data-processing algorithms, each of which makes its own assumptions about the data. Consequently, although qPCR has frequently been proclaimed a touchstone or a gold standard, in practice this “standard” is a variable one, and the reporting of results requires considerable sophistication of analysis and interpretation (43).

3. Research vs Diagnostic Applications

Applications of qPCR technology can be broadly divided into research and diagnostic applications. Research applications usually analyze a wide range of targets with a fairly low throughput and many different sample types. The main parameters that need to be addressed relate to assay analytical sensitivity

and specificity, which in this context refer to how many target copies the assay can detect and whether the no-template controls (NTCs) are reliably negative, respectively.

In contrast, diagnostic applications usually analyze a limited number of targets, but require high-throughput protocols that are targeted at only a few sample types. Although all of the considerations that apply to research applications also apply to diagnostic assays, clinical-diagnostic assays have a number of additional requirements that need to be considered. These requirements include information on analytical sensitivity and specificity that in this context refers to how often the assay returns a positive result when a target is present and how often it is negative in the absence of the target. Furthermore, the accuracy and precision within and between laboratories is often monitored by external QC programs. Additional clinical laboratory requirements include criteria for generating reportable results, whether repeated measurements are made on samples, data on the resolution of false-positive/false-negative data, and the similarity of results from multiple laboratories that use the same and different technologies. Thus far, only a couple of interlaboratory comparisons have been performed, and both of these studies emphasized the need for standardization of qPCR diagnostic assays (44, 45). Another interlaboratory exercise is planned within the European Framework 7 project: SPIDIA (Standardisation and Improvement of Generic Pre-analytical Tools and Procedures for In-Vitro Diagnostics; <http://www.spidia.eu>).

4. Sample Acquisition, Handling, and Preparation

Sample acquisition constitutes the first potential source of experimental variability, especially for experiments targeting RNA, because mRNA profiles are easily perturbed by sample-collection and -processing methods. There is some suggestion that fresh tissue can be stored on ice without major effects on RNA quality and concentration (46), but although this supposition may be true for some mRNAs and tissues, it may not be universally applicable. Hence, it is better to be cautious. Consequently, it is important to report in detail where the tissue sample was obtained and whether it was processed immediately. If the sample was not processed immediately, it is necessary to report how it was preserved and how long and under what conditions it was stored.

A brief description of the sample is also essential. For example, microscopical examination of a tumor biopsy will reveal what percentage of the biopsy is made up of tumor cells, and this information should be reported.

Nucleic acid extraction is a second critical step. Extraction efficiency depends on adequate homogenization, the type of sample (e.g., in situ tissue vs log-phase cultured cells), target density, physiological status (e.g., healthy, cancerous, or necrotic), genetic complexity, and the amount of biomass processed. Therefore, it is necessary that details of the nucleic acid-extraction method be provided and that the methods used for measuring nucleic acid concentration and assessing its quality be described. Such details are particularly crucial for RNA extracted from fresh frozen laser-microdissected biopsy samples, because variations in tissue-preparation procedures have a substantial effect on both RNA yield and quality (47).

5. QC of Nucleic Acids

5.1. RNA SAMPLES

Quantification of RNA in the extracted samples is important, because it is advisable that approximately the same amounts of RNA be used when comparing different samples. There are several quantification procedures in common use, however, including spectrophotometry (NanoDrop; Thermo Scientific), microfluidic analysis (Agilent Technologies' Bioanalyzer, Bio-Rad Laboratories' Experion), capillary gel electrophoresis (Qiagen's QIAxcel), or fluorescent dye detection (Ambion/Applied Biosystems' RiboGreen). The methods produce different results, making it unwise to try to compare data obtained with the different methods (48). The preferred method for quantifying RNA uses fluorescent RNA-binding dyes (e.g., RiboGreen), which are best for detecting low target concentrations. In any case, it is advisable to measure all samples with a single method only and to report this information.

It is also important to test for and report the extent of genomic-DNA contamination and to record the threshold cutoff criteria for the amounts of such contamination that are tolerable. It is essential to report whether the RNA sample has been treated with DNase (including the type of DNase used and the reaction conditions) and to report the results from a comparison of C_q s obtained with positive and no-reverse transcription controls for each nucleic acid target.

It is also essential to document the quality assessment of RNA templates. The only situation in which this requirement does not apply is when the quantity of total RNA extracted is too low to permit quality assessment. This situation arises when RNA is extracted from single cells, plasma, other cell-free body fluids, some laser-captured samples, or clarified tissue culture medium. It also applies in cases in which extraction and RT-qPCR steps are performed as a continuous, single-tube experiment. Key information to report includes data on RNA quantity, integrity, and the absence of

reverse transcription or PCR inhibitors. It is worth remembering that RNA degrades markedly *in vivo*, owing to the natural regulation of mRNAs in response to environmental stimuli (49). This source of RNA degradation is beyond the control of the researcher; one of its manifestations is that even high-quality RNA samples can show differential degradation of individual mRNAs.

The A_{260}/A_{280} ratio must be measured in a buffer at neutral pH, but such measurement is not sufficient if the nucleic acid is to be used for quantitative analysis, especially when the aim is to measure minor differences (<10-fold) in cellular mRNA concentrations. The absorbance ratio does provide an indication of RNA purity, because the presence of DNA or residual phenol alters the ratio. Instead, one should provide gel electrophoresis evidence at the least or, better yet, results from a microfluidics-based rRNA analysis (50) or a reference gene/target gene 3':5' integrity assay (51). The advantage of the use of a Bioanalyzer/Experion system to calculate an RNA integrity number or an RNA quality indicator number is that these measures provide quantitative information about the general state of the RNA sample. It is important to bear in mind, however, that these numbers relate to rRNA quality and cannot be expected to be an absolute measure of quality. Use of a 3':5' assay requires that the PCR efficiencies of both assays be virtually identical (51) and not be subject to differential inhibition. This assay also necessitates the establishment of a threshold criterion that delineates the RNA quality sufficient to yield reliable results. Ideally, the assay should target a panel of "integrity reference genes," probably without introns, with a 3':5' threshold ratio of approximately 0.2–5. Clearly, further work is required to generate a universally applicable, cost-effective, and simple protocol for assessing RNA integrity.

Inhibition of reverse-transcription activity or PCR should be checked by dilution of the sample (preferably) or use of a universal inhibition assay such as SPUD (52, 53). If the RNA sample is shown to be partially degraded, it is essential that this information be reported, because the assay's sensitivity for detecting a low-level transcript may be reduced and relative differences in the degradation of transcripts may produce incorrect target ratios.

5.2. DNA SAMPLES

In general, degradation is much less of an issue with DNA; however, it is important to be able to assess the extent of DNA degradation for forensic applications, i.e., in cases in which harsh environmental conditions at scenes of crimes or mass disasters or at sites involving missing-person cases may have degraded the chemical structure of DNA. The small amplicon size of qPCR

assays helps to minimize assay-related problems, but methods have been developed that provide a quantitative measurement of DNA quality (54) and should be considered for such specialized purposes.

The potential for inhibition is a more generally applicable variable that must be addressed in a publication, and it is important to ensure that no inhibitors copurified with the DNA will distort results, e.g., pathogen detection and their quantification (55). Although such approaches such as spiking samples with positive controls (52) can be used to detect inhibition, different PCR reactions may not be equally susceptible to inhibition by substances copurified in nucleic acid extracts (56, 57). Consequently, it is better to routinely use dilutions of nucleic acids to demonstrate that observed decreases in C_{qs} or copy numbers are consistent with the anticipated result and to report these data.

6. Reverse Transcription

The reverse-transcription step introduces substantial variation into an RT-qPCR assay (58, 59). Hence, it is essential that a detailed description of the protocol and reagents used to convert RNA into cDNA be provided. This documentation must include the amount of RNA reverse-transcribed, priming strategy, enzyme type, volume, temperature, and duration of the reverse-transcription step. It is recommended that the reverse-transcription step be carried out in duplicate or triplicate and that the total RNA concentration be the same in every sample (58).

7. qPCR

The following information must be provided for qPCR assays: database accession numbers of each target and reference gene, the exon locations of each primer and any probe, the sequences and concentrations of each oligonucleotide, including the identities, positions, and linkages of any dyes and/or modified bases. Also required are the concentration and identity of the polymerase, the amount of template (DNA or cDNA) in each reaction, the Mg^{2+} concentration, the exact chemical compositions of the buffer (salts, pH, additives), and the reaction volume. The investigators must also identify the instrument they used and document all of the PCR cycling conditions. Because the consumables used affect thermal cycling, it is necessary to identify the use of single tubes, strips, or plates, and their manufacturers. The degree of transparency of the plasticware used, e.g., white or clear, is also important, because different plastics exhibit substantial differences in fluorescence reflection and sensitivity (60). When plates are used, the method of sealing (heat bonding

vs adhesives) can affect the evaporation of samples at the plate perimeter and should therefore be documented.

Because PCR efficiency is highly dependent on the primers used, their sequences must be published. This requirement is perfectly feasible even with commercial primers, because there is a precedent for companies to make their primer and probe sequences available (http://www.primerdesign.co.uk/research_with_integrity.asp).

In addition, submission to public databases such as RTprimerDB is strongly encouraged; over time, these databases could become universal clearinghouses.

7.1. SECONDARY STRUCTURE

The structure of the nucleic acid target (e.g., stem and loop secondary RNA structure) has a substantial impact on the efficiency of reverse transcription and the PCR. Therefore, the positions of primers, probes, and PCR amplicons must take the folding of RNA templates into consideration. Sequences should be checked with nucleic acid-folding software, e.g., mfold for DNA (<http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi>) or RNA (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1-2.3.cgi>). Ideally, the folding structures should be made available to reviewers.

7.2. SPECIFICITY

In silico tools such as BLAST or equivalent specificity searches are useful for assay design. Any appreciable homology to pseudogenes or other unexpected targets should be documented and provided as aligned sequences for review; however, specificity must be validated empirically with direct experimental evidence (electrophoresis gel, melting profile, DNA sequencing, amplicon size, and/or restriction enzyme digestion).

Algorithms for predicting an oligonucleotide's melting temperature (T_m) are useful for initial design, but the practical optimum temperature for annealing must be determined experimentally. Although primer optimization has become unfashionable, it is clear that poor annealing optimization has a large effect on assay quality (51). A marked presence of primer dimers produces a lower PCR efficiency in probe-based assays and may generate false positives in assays based on SYBR Green I. Some evidence for primer optimization should be provided to reviewers, ideally in the form of annealing temperature or Mg^{2+} gradients, and be presented as C_q values, plots of fluorescence vs cycle number, and/or melting curves (61).

7.3. CONTROLS AND QUANTIFICATION CALIBRATORS

In addition to the no-reverse transcription control in RT-qPCR assays mentioned above, additional controls

and/or quantification calibrators are required for all qPCR reactions. NTCs detect PCR contamination when probes are used and can also distinguish unintended amplification products (e.g., primer dimers) from the intended PCR products in SYBR Green I reactions. NTCs should be included on each plate or batch of samples, and conditions for data rejection be established. For example, NTCs with $C_q \geq 40$ could be ignored if the C_q for the lowest concentration unknown is 35.

Positive controls in the form of nucleic acids extracted from experimental samples are useful for monitoring assay variation over time and are essential when calibration curves are not performed in each run.

Quantification calibrators may be purified target molecules, such as synthetic RNA or DNA oligonucleotides spanning the complete PCR amplicon, plasmid DNA constructs, cDNA cloned into plasmids, RNA transcribed in vitro, reference RNA pools, RNA or DNA from specific biological samples, or internationally recognized biological standards (as they become available). Dilutions should be carried out into defined concentrations of carrier tRNA (yeast or *Escherichia coli* at 10–100 ng/ μ L). For detection of human pathogens, calibrators can be diluted into negative control sample RNA or DNA, or they can be diluted into healthy human plasma, after which lysis may be carried out in the presence of carrier tRNA. Serial dilutions of a particular template can be prepared as stock solutions that resist several freeze-thaw cycles. A fresh batch should be prepared when a C_q shift of 0.5–1.0 is detected. Alternatively, solutions for calibration curves can be stored for a week at 4 °C and then discarded.

For diagnostic assays, the qPCR should include an independently verified calibrator, if available, that lies within the linear interval of the assay. Positive and negative extraction controls are also recommended.

7.4. ASSAY PERFORMANCE

The following assay performance characteristics must be determined: PCR efficiency, linear dynamic range, LOD, and precision.

7.4.1. PCR efficiency. Robust and precise qPCR assays are usually correlated with high PCR efficiency. PCR efficiency is particularly important when reporting mRNA concentrations for target genes relative to those of reference genes. The $\Delta\Delta C_q$ method is one of the most popular means of determining differences in concentrations between samples and is based on normalization with a single reference gene. The difference in C_q values (ΔC_q) between the target gene and the reference gene is calculated, and the ΔC_q s of the different samples are compared directly. The 2 genes must be amplified with comparable efficiencies for this com-

parison to be accurate. The most popular method is not necessarily the most appropriate, however, and alternative, more generalized quantitative models have been developed to correct for differences in amplification efficiency (62) and to allow the use of multiple reference genes (30).

PCR amplification efficiency must be established by means of calibration curves, because such calibration provides a simple, rapid, and reproducible indication of the mean PCR efficiency, the analytical sensitivity, and the robustness of the assay. Amplification efficiency should be determined from the slope of the log-linear portion of the calibration curve. Specifically, $\text{PCR efficiency} = 10^{-1/\text{slope}} - 1$, when the logarithm of the initial template concentration (the independent variable) is plotted on the x axis and C_q (the dependent variable) is plotted on the y axis. The theoretical maximum of 1.00 (or 100%) indicates that the amount of product doubles with each cycle. Ideally, the CIs or SEs of the means of estimated PCR efficiencies are reported from replicated calibration curves.

Calibration curves for each quantified target must be included with the submitted manuscript so that this information can be made available to the reviewers; slopes and y intercepts derived from these calibration curves must be included with the publication. Differences in PCR efficiency will produce calibration curves with different slopes. As a consequence, differences between the C_q values of the targets and the references will not remain constant as template amounts are varied, and calculations of relative concentrations will be inaccurate, yielding misleading results.

C_q values >40 are suspect because of the implied low efficiency and generally should not be reported; however, the use of such arbitrary C_q cutoffs is not ideal, because they may be either too low (eliminating valid results) or too high (increasing false-positive results) (26).

7.4.2. Linear dynamic range. The dynamic range over which a reaction is linear (the highest to the lowest quantifiable copy number established by means of a calibration curve) must be described. Depending on the template used for generating calibration curves, the dynamic range should cover at least 3 orders of magnitude and ideally should extend to 5 or 6 \log_{10} concentrations. The calibration curve's linear interval must include the interval for the target nucleic acids being quantified. Because lower limits of quantification are usually poorly defined, the variation at the lowest concentration claimed to be within the linear interval should be determined. Correlation coefficients (r^2 values) must be reported, and, ideally, CIs should be provided through the entire linear dynamic range.

7.4.3. LOD. The LOD is defined as the lowest concentration at which 95% of the positive samples are detected. In other words, within a group of replicates containing the target at concentrations at the LOD, no more than 5% failed reactions should occur. Low-copy PCRs are stochastically limited, and LODs of <3 copies per PCR are not possible. If multiple reactions are performed, however, accurate quantification of lower concentrations can be obtained via digital PCR (29, 63, 64). Indeed, concentration calibrators can be checked by limiting dilution to show that the percentage of failed and successful reactions follows a Poisson distribution.

7.4.4. Precision. There are many explanations for variation in qPCR results, including temperature differences affecting the completion of annealing and/or denaturation, concentration differences introduced by pipetting errors, and stochastic variation. Precision in qPCR typically varies with concentration, decreasing with the copy number. Ideally, intraassay variation (repeatability) should be displayed in figures as SD error bars or as CIs on calibration curves with replicate samples. CVs should not be used with C_q s (29) but can be used to express the variance in copy numbers or concentrations. This technical variation should be distinguished from biological variation. Biological replicates can directly address the statistical significance of differences in qPCR results between groups or treatments. For diagnostic assays, it may also be necessary to report interassay precision (reproducibility) between sites and different operators.

7.5. MULTIPLEX qPCR

The ability to multiplex greatly expands the power of qPCR analysis (65, 66), particularly when applied to the simultaneous detection of point mutations or polymorphisms (67). Multiplexing requires the presentation of evidence demonstrating that accurate quantification of multiple targets in a single tube is not impaired, i.e., that assay efficiency and the LOD are the same as when the assays are run in uniplex fashion. This concern is of particular importance when targets of appreciably lower abundance are coamplified with highly abundant targets.

8. Data Analysis

Data analysis includes an examination of the raw data, an evaluation of their quality and reliability, and the generation of reportable results. Various data-collection and -processing strategies have been described, and a systematic evaluation has revealed that qPCR data-analysis methods differ substantially in their performance (68).

Detailed information on the methods of data analysis and confidence estimation is necessary, together with specification of the software used. The methods of identifying outliers and the disposition of such data must be specified. Documenting assay precision requires identification of the statistical methods used to evaluate variances (e.g., 95% CIs) and presentation of the corresponding concentrations or C_q values. Such information should include both repeatability and reproducibility data, if available. As discussed above, reporting of CVs for C_q s is inappropriate (29), because C_q s will always be lower (and therefore potentially misleading) than CVs calculated for copy numbers. Information must be provided on the methods used for assessing accuracy, including the statistical significance of reported differences between groups.

8.1. NORMALIZATION

Normalization is an essential component of a reliable qPCR assay because this process controls for variations in extraction yield, reverse-transcription yield, and efficiency of amplification, thus enabling comparisons of mRNA concentrations across different samples. The use of reference genes as internal controls is the most common method for normalizing cellular mRNA data; however, although the use of reference genes is commonly accepted as the most appropriate normalization strategy (69), their utility must be experimentally validated for particular tissues or cell types and specific experimental designs. Unfortunately, although there is an increased awareness of the importance of systematic validation and although the potentially highly misleading effects of the use of inappropriate reference genes for normalization are widely known, these considerations also are still widely disregarded (70). Consequently, many molecular analyses still contain qPCR data that are poorly normalized.

Normalization involves reporting the ratios of the mRNA concentrations of the genes of interest to those of the reference genes. Reference gene mRNAs should be stably expressed, and their abundances should show strong correlation with the total amounts of mRNA present in the samples.

Normalization against a single reference gene is not acceptable unless the investigators present clear evidence for the reviewers that confirms its invariant expression under the experimental conditions described. The optimal number and choice of reference genes must be experimentally determined and the method reported (71–73).

8.2. VARIABILITY

The inherent variability of biological systems may rival or exceed experimental differences between groups. This variation is often observed when many biological

replicates are used to increase the statistical significance of the experiment. Although differences between biological replicates may be large, sufficient numbers may allow smaller experimental differences to be discerned. A recent publication provides a textbook example for handling such data and how to salvage biologically meaningful data from assays subject to high biological variation (74). Many factors contribute to experimental variation and influence the number of biological replicates necessary to achieve a given statistical power. Consequently, power analysis is useful for determining the number of samples necessary for valid conclusions.

8.3. QUALITATIVE ANALYSIS

The use of the PCR to detect merely the presence of a nucleic acid template, rather than to quantify it accurately, is referred to as qualitative PCR, which is widely used in pathogen diagnostics. The problem with qualitative/quantitative stratification of PCR methods is that an accurate yes/no answer requires information about the low-end sensitivity of the PCR assay. Consequently, even a qualitative assay should provide information about the assay's performance characteristics, especially with respect to the points discussed in sections 7.4.2. and 7.4.3.

Conclusions

The necessity for ensuring quality-assurance measures for qPCR and RT-qPCR assays is well recognized (25, 44, 75–86). The main difference between qPCR and conventional (legacy) PCR assays is the expectation of the former's potential to quantify target nucleic acids accurately. This difference must be clearly recognized, and one cannot assume that legacy PCR assays can translate directly into the qPCR format. Table 1 provides a checklist for authors preparing a report of a qPCR study. Items deemed essential (E) are required to allow reviewers to evaluate the work and other investigators to reproduce it. Items considered desirable (D) are also important and should be included if possible, but they may not be available in all cases. Certainly, it is important to apply common sense: Compliance with all items on the checklist is not necessary for initial screening of expression signatures targeting hundreds of targets. Once a more limited set of targets (fewer than 20) has been identified, however, assay performance should be described as detailed by the checklist, which is hosted on <http://www.rdml.org/miqe/>.

In summary, the purpose of these guidelines is 3-fold:

1. To enable authors to design and report qPCR experiments that have greater inherent value.

2. To allow reviewers and editors to measure the technical quality of submitted manuscripts against an established yardstick.
3. To facilitate easier replication of experiments described in published studies that follow these guidelines.

As a consequence, investigations that use this widely applied technology will produce data that are more uniform, more comparable, and, ultimately, more reliable.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: J. Hellemans, Biogazelle; J. Vandesompele, Biogazelle; C.T. Wittwer, Idaho Technology.

Consultant or Advisory Role: R. Mueller, DermTech International.

Stock Ownership: R. Mueller, Sequenom; C.T. Wittwer, Idaho Technology.

Honoraria: None declared.

Research Funding: S.A. Bustin, Bowel and Cancer Research, registered charity number 1119105; J. Hellemans, Fund for Scientific Research Flanders; M. Kubista, grant agency of the Academy of Sciences, Czech Republic (grants IAA500520809 and AV0250520701); C.T. Wittwer, ARUP Institute for Clinical and Experimental Pathology and Idaho Technology.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Notas



En los últimos años, la técnica de Transcripción Reversa seguida de una Reacción en Cadena de la Polimerasa Cuantitativa (RT-qPCR) se convirtió en una metodología de referencia para determinar los niveles de expresión génica debido a su precisión, sensibilidad, exactitud, especificidad, reproducibilidad y robustez. Además de su óptima performance analítica, presenta una amplia variedad de aplicaciones, lo que en conjunto determinó su amplia y rápida difusión. Sin embargo para el usuario novato se presenta como una metodología difícil de utilizar, fundamentalmente debido a la complejidad metodológica en la ejecución y en el procesamiento de los datos. En un intento por organizar el estudio que permita la mejor difusión y desarrollo de la técnica, la comunidad científica internacional generó un consenso sobre la información necesaria para realizar la misma denominado The MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments). Nuestro objetivo es poder brindarles herramientas y asesoramiento para que, a lo largo del curso basados en la propuesta de la Guía MIQE, desarrollen un protocolo posible de RT-qPCR que permita la puesta a punto o la optimización de una determinación.

Juanito remontando su barrilete (1973)
Antonio Berni. Oleo y collage sobre madera



ISBN 978-987-25098-4-0

